Histamine cloning protocol

First step:
Primers arrived => Dilute them as discribed from the company.

Seconed step:

1. PCR reaction for a subset of variants to make sure everything is working.

control- pcr reaction without DNA template

variant	F-primer	F-primer (Amount)	R-primer	R-primer (Amount)	Tm
1	His_BG_For		His_AA_Rev		64.2
2	His_BA_For		His_AA_Rev		64.2
3	His_BB_For		His_AA_Rev		64.2
4	His_BC_For		His_AA_Rev		64.2
11	His_BB_For		His_AB_Rev		60.8
control	His_BG_For		His_AA_Rev		64.2

Component	Volume	control
5x PCR Buffer	10 µl	10 µl
dNTPs (10mM)	1 µl	1 µl
Forward primer [10 µM]	2.5 µl	2.5µl
Reverse primer [10 µM]	2.5 µl	2.5µl
Template DNA - Tar2 CM (2- 10 ng)	1 µl	-
Phusion DNA Polymerase	0.5 µl	0.5
UPW	32.5 µl	33.5 µl
Total	50	50

Step	Temperature	Time
Initial denaturation	98	30 sec
Denaturation	98	10 sec
Annealing	grad of 64, 61	30 sec
Extension	72	2 min
Final extension	72	10 min
Hold	4	hold

30 cycles

2. Gel electrophoresis (p.10-12) - 1% agar (500 bp to 10 kb) [50 min]

   5 µl of ladder

   5 µl of sample + 1 µl dye

Ladder	1	2	3	4	11	control	Ladder

Expected length-

THE WHOLE TAR- 3930bp

3. PCR reaction for the rest of variants.

variant	F-primer	F-primer (Amount)	R-primer	R-primer (Amount)	Tm
5	His_BD_For		His_AA_Rev		64.2
6	His_BD_For		His_AA_Rev		64.2
7	His_BE_For		His_AA_Rev		64.2
8	His_BG_For		His_AA_Rev		64.2
9	His_BG_For		His_AA_Rev		64.2
10	His_BF_For		His_AA_Rev		64.2
control	His_BG_For		His_AA_Rev		64.2

Component	Volume	control
5x PCR Buffer	10 µl	10 µl
dNTPs (10mM)	1 µl	1 µl
Forward primer [10 µM]	2.5 µl	2.5µl
Reverse primer [10 µM]	2.5 µl	2.5µl
Template DNA - Tar2 CM (2- 10 ng)	1 µl	-
Phusion DNA Polymerase	0.5 µl	0.5
UPW	32.5 µl	33.5 µl
Total	50	50

The program is as follows:

Step	Temperature	Time
Initial denaturation	98	30 sec
Denaturation	98	10 sec
Annealing	64	30 sec
Extension	72	2 min
Final extension	72	10 min
Hold	4	hold

30 cycles

4. Gel electrophoresis (p.10-12) - 1% agar (500 bp to 10 kb) [50 min]

   5 µl of ladder

   5 µl of sample + 1 µl dye

Ladder	5	6	7	8	9	10	control	Ladder

Expected length-

THE WHOLE TAR- 3930bp

5. Purification of PCR product for all 11 varients (p. 13-14) [15 min]

   Equal volume = 45 µl

6. DpnI for DNA (in order to digest methylated plasmid)

Materials:

- Restriction Enzymes (DpnI)
- NEB buffer (CutSmart buffer)
- Molecular biology water

Procedure:
Set up the following reaction a described below:

component	Volume(ul)
DNA	45
10X CutSmart Buffer	6
DpnI	1
UPW	8
Total	60ul

- 1hr incubation in 37C.
- Purification of digestation product

7. Phosphorylation

	ligation	Control- no phosph.
T4 DNA ligase buffer	2.5 µl	2.5
T4 kinase (PNK)	1 µl	-
DNA	X~100 ng= 3ul	3
PEG 4000	2.5 µl (5%)	-
MB H2O	19-X µl	19.5
Total	**25 µl**	**25µl**

Incubate at 37°C for 30 min
Heat inactivation prior to ligation 65°C for 20 min
Cool on ice for 10 min

8. Self Ligation

Add to the solution (from phosphorylation step after cooling) 1 µl T4 DNA ligase
Incubate for 2 hr at 30°C or overnight in 16°C
Ligation is ready for transformation, take 5µl (heat shock only).

9. Transformation to Top10
10. Mini prep for positive colonies
11. sequencing

Third step:

1. second PCR reaction for a subset of variants to make sure everything is working.

variant	F-primer	F-primer (Amount)	R-primer	R-primer (Amount)	Tm
1	His_DA_For		His_CA_Rev		65.4
3	His_DB_For		His_CC_Rev		67.2
5	His_DC_For		His_CE_Rev		64.1
10	His_DB_For		His_CG_Rev		65.3
11	His_DD_For		His_CA_Rev		63.5
control	His_DB_For		His_CA_Rev		63.5

Component	**Volume**	**control**
5x PCR Buffer	10 µl	10 µl
dNTPs (10mM)	1 µl	1 µl
Forward primer [10 µM]	2.5 µl	2.5µl
Reverse primer [10 µM]	2.5 µl	2.5µl
Template DNA - prev cloning (2- 10 ng)	1 µl	-
Phusion DNA Polymerase	0.5 µl	0.5
UPW	32.5 µl	33.5 µl
Total	50	50

The program is as follows:

Step	Temperature	Time
Initial denaturation	98	30 sec
Denaturation	98	10 sec
Annealing	grad 63-67	30 sec
Extension	72	2 min
Final extension	72	10 min
Hold	4	hold

30 cycles

2. Gel electrophoresis (p.10-12) - 1% agar (500 bp to 10 kb) [50 min]

5 µl of ladder

5 µl of sample + 1 µl dye

Ladder	1	3	5	10	11	control	Ladder

Expected length-

THE WHOLE TAR- 3930bp

3. Second PCR reaction for rest of variants.

variant	F-primer	F-primer (Amount)	R-primer	R-primer (Amount)	Tm
2	His_DB_For		His_CB_Rev		64.9
4	His_DB_For		His_CD_Rev		63.3
6	His_DB_For		His_CE_Rev		63.4
7	His_DB_For		His_CC_Rev		67.2
8	His_DB_For		His_CA_Rev		63.5
9	His_DB_For		His_CF_Rev		63.3
control	His_DB_For		His_CA_Rev		63.5

Component	**Volume**	**control**
5x PCR Buffer	10 µl	10 µl
dNTPs (10mM)	1 µl	1 µl
Forward primer [10 µM]	2.5 µl	2.5µl
Reverse primer [10 µM]	2.5 µl	2.5µl
Template DNA - prev cloning (2- 10 ng)	1 µl	-
Phusion DNA Polymerase	0.5 µl	0.5
UPW	32.5 µl	33.5 µl
Total	50	50

The program is as follows:

Step	Temperature	Time

Initial denaturation	98	30 sec
Denaturation	98	10 sec
Annealing	grad 63-67	30 sec
Extension	72	2 min
Final extension	72	10 min
Hold	4	hold

30 cycles

3. Gel electrophoresis (p.10-12) - 1% agar (500 bp to 10 kb) [50 min]

   5 µl of ladder

   5 µl of sample + 1 µl dye

Ladder	2	4	6	7	8	9	control	Ladder

Expected length-

THE WHOLE TAR- 3930bp

4. Purification of PCR product (p. 13-14)

   Equal volume = 45 µl

5. DpnI for DNA (in order to digest methylated plasmid)

Materials:

- Restriction Enzymes (DpnI)
- NEB buffer (CutSmart buffer)
- Molecular biology water

Procedure:

Set up the following reaction a described below:

component	Volume(ul)
DNA	45
10X Buffer	6
DpnI	1
UPW	8
Total	60ul

- 1hr incubation in 37C.

6. Purification of digestation product
7. Ligation

Component	Volume	control
10x T4 DNA Ligase Buffer	2 µl	2 µl
Template DNA - Tar1 CM (25-50 ng)	?? µl	-
T4 DNA Ligase	1 µl	1 µl
MBW	Y µl	17 µl
Total	20	20

8. Transformation to UU
9. Mini prep for positive colonies
10. sequencing
11. Chemotaxis checks: Swarming assay (?), microscope